

Full Length Research Paper

## Effects of immunoglobulin Y (IgY) serum against plaque bacteria

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Dental caries is an infectious disease caused by plaque bacteria, especially *Streptococcus mutans*. Many studies were conducted to develop methods or material for dental caries prevention. It was using passive immunization that will produce specific antibodies. Yolk immunoglobulin (IgY) is antibody in the serum and egg yolk. This study aimed to determine the effect of IgY serum against the growth of dental plaque bacterial in vitro. In the long term, it is expected IgY specific *S. mutans* can be as one of material could prevent dental caries topically. Immunization in the chicken was performed using *S. mutans* antigen. Immunoglobulin Y (IgY) specific *S. mutans* was collected from blood serum of Hysex Brown chicken. Antibacterial effect of IgY serum on the growth of bacteria that cause dental plaque (*Streptococcus alpha*, *Staphylococcus aureus*, and *S. mutans*) was done using diffusion method. Sample was performed on 10 plate each bacteria. Data inhibition zone were analyzed using ANOVA and LSD. There was no inhibition zone of IgY serum on the growth of *S. alpha* and *S. aureus* while positive results obtained against *S. mutans*. Inhibition zone increased according increasing the concentration of IgY. ANOVA test showed significant differences the effect of concentration IgY serum on the inhibition zones of *S. mutans* ( $p < 0.05$ ). A significant difference ( $p < 0.05$ ) from the LSD was obtained in the comparison between the concentration of IgY, IgY serum and controls. In conclusion, IgY serum that immunized *S. mutans* has antibacterial effect against *S. mutans* only.

**Key words:** Yolk immunoglobulin (IgY), chicken serum, *Streptococcus alpha*, *Staphylococcus aureus*, *Streptococcus mutans*

### INTRODUCTION

Dental caries is an infectious disease which can be found in almost all people in the world. Dental caries is known as a multifactorial disease. *Streptococcus mutans* is known as dental caries bacteria. This disease has been known

since the nineteenth century, from the textbook Black noted that dental caries is defined simply as the chemical dissolution of calcium in teeth by lactic acid, followed by decomposition of the organic matrix of dentin.

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In the mid-nineteenth century, caries observed in Europeans was severe. At that time a lot of people aged around 14 year old had lost teeth due to dental caries which incriminate pulp (Fejerskov and Kidd, 2003).

Until now, dental caries condition is still quite alarming, it can be seen from the result of the Household Health Survey (SKRT) in 2015 (Ministry of Health, 2015) that the prevalence of caries disease in Indonesia is still showing significant number which is 90.05%. Problems which is still occurring now is whether dental caries as an infectious disease can be prevented or not. Fejerskov and Kidd (2003) expressed their opinion that the formation of caries cavity can be prevented through the control of process which involving caries however metabolic fluctuations in biofilms cannot be prevented. Various efforts to prevent the formation of dental caries cavity have been performed, such as flour application in drinking water as well as in toothpaste. Also immunization against dental caries though it is still in the research stage.

One of the attempts to immunize against dental caries is producing antibodies that will be expected to be able to prevent caries cavity. Yolk Immunoglobulin (IgY) is immunoglobulin found in chicken egg which is derived from the blood as a result of exposure to antigen. The use of specific IgY is known to be beneficial for treatments or therapy. Hatta et al. (1990) have found a simple method for the isolation of yolk antibodies. The production of specific IgY against *S. mutans* through passive immunization has been carried out by Hatta et al. (1994) and Poetri and Soejoedono (2006).

Normal flora in the oral cavity are known to be complex and about 350 species of bacteria have been cultured, however still a lot of species of bacteria that are still in the stage of identification. *Streptococcus* genus in the oral cavity consists of 4 main species which are *mutans*, *salivarius*, *anginosus* and *mythic*. *Streptococcus* can be classified according to degrees of hemolysis on blood agar (Samaranayake, 2001).

Dental plaque is composed by micro-organisms, especially bacteria. One gram plaques are composed of about  $2 \times 10^{11}$  bacteria. Early bacteria colonization on the tooth surface occurs and within a few hours the bacteria can already be found on the pellicle. The bacteria that hold the initial colonization of the pellicle on the surface of the oral tissues is especially facultative Gram-positive bacteria such as *Actinomyces viscosus* and *Streptococcus sanguinis* (Newman et al., 2002). According to Michalek and Mc Ghee (1982) *Streptococcus alpha* or *Streptococcus viridans* were also known as the dominant bacteria in early plaque formation and this bacteria is to facilitate the colonization of other bacteria including anaerobic bacteria which has a big role in periodontal disease. In addition, *Streptococcus* also produces histolytic enzymes and toxic substances that can damage tissues.

Growth of bacteria in the oral cavity is important to know because the disease originated from the change of bacteria from normal flora to a pathogenic bacteria. Preliminary research on the effectiveness of egg yolk antibodies (IgY) as an anti-caries ingredient through the hydrophobicity test against *S. mutans* has been done by Azis et al. (2013) through Research - Student Creativity Program (PKM-P). Mechanism of IgY antibodies against bacterial growth is important to be learned because it is expected to be a material that is able to maintain homeostasis of microflora in the mouth so that in the long-term can be used as a preventive to prevent dental caries. Also about the effects of yolk immunoglobulin (IgY) serum on the growth of bacteria of dental plaque bacteria *in vitro* (Studies on Chicken Serum which is immunized by *Streptococcus mutans*). Therefore, this study aimed to determine the effects of yolk immunoglobulin (IgY) serum towards the growth of dental plaque bacteria *in vitro* so that in the long term it is expected to be used as prevention of dental caries.

## MATERIALS AND METHODS

This study was a pure laboratory experimentation. The procedure of this study was approved by the Ethics Committee, Faculty of Dentistry, Universitas Gadjah Mada. The study was conducted at the Laboratory of Microbiology, Faculty of Veterinary, Universitas Gadjah Mada.

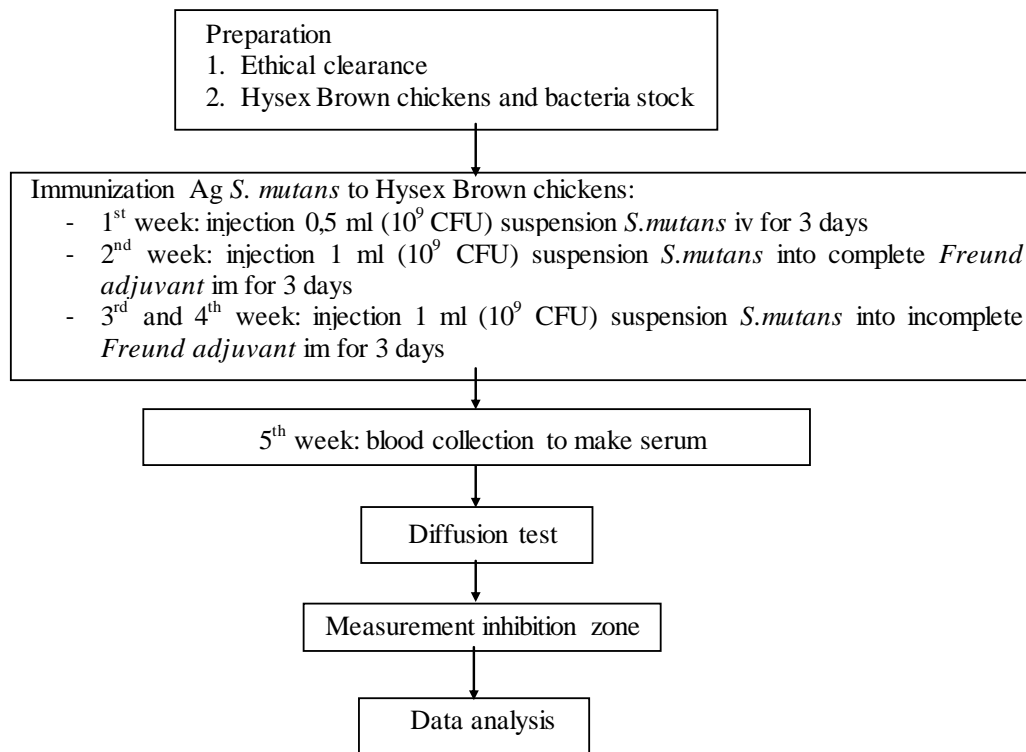
### Immunization of experimental animals

Four Hysex Brown chickens aged 18-24 weeks were immunized with 0.5 ml ( $10^9$  CFU) suspension of *S. mutans* intravenously for three consecutive days. Followed by injection of 1 ml ( $10^9$  CFU) suspension of bacteria into complete Freund's adjuvant at week 2, and 1 ml ( $10^9$  CFU) suspension of bacteria *S. mutans* into incomplete Freund's adjuvant at week 3 and 4 intramuscularly. After 1 week serum was collected.

### Difussion test

*S. alpha* and *Staphylococcus aureus* were from stock bacteria in the Laboratory of Microbiology, Faculty of Veterinary, Universitas Gadjah Mada. *S. mutans* ATCC 25175 was procured from Dr. Yulita Kristanti, Department Conservative Dentistry, Universitas Gadjah Mada. *S. mutans* has been subcultured and re-identified in the Laboratory of Microbiology, Faculty of Medicine, Universitas Gadjah Mada.

*S. alpha*, *S. aureus*, and *S. mutans* were cultured on blood plates media then incubated for 18-24 h at 37°C. Colony of bacteria was taken from the subculture media and then put into 3 ml of liquid Brain Heart Infusion (BHI) media and incubated for 18 hours in an incubator at 37°C. The resulting bacterial suspension in BHI media was compared to the turbidity of 0.5 McFarland standard which is equivalent to  $10^8$  CFU/ml. Disk diffusion test were done by measuring the diameter of the clear zone as an indication of bacterial growth inhibitory response by an antibacterial compound. Bacterial growth was observed to see the formation of inhibition zones around the pits. Diameter of inhibition zones that was clear



**Figure 1.** Flowchart of study.

area (there was no bacterial growth) around the wells were measured using a caliper.

#### Statistical analysis

All data were analyzed using normality test and homogeneity of variance test, then analyzed using statistical tests Analysis of Variance (ANOVA) and post hoc Least Significant Difference (LSD) (Figure 1).

## RESULTS

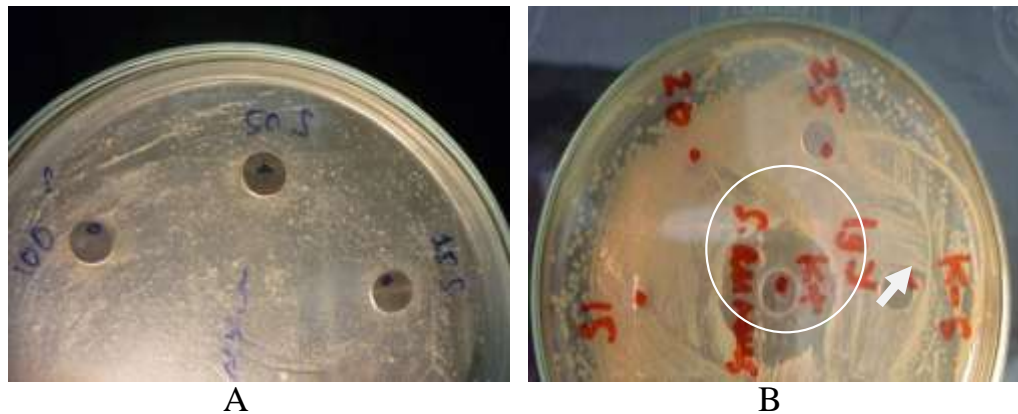
Positive result of IgY was detected using Agar Gel Precipitation Test (AGPT), a positive reaction was marked by the precipitation line between the antigen pit and antibody. Azis et al. (2013) resulted that the positive result of IgY in chicken egg yolk were obtained at week 5 after immunization so that the study was conducted after taking the serum at week 5 after immunization. IgY antibacterial activity testing of some IgY serum concentration levels that have been immunized with *S. mutans* were done against the dental plaque bacteria. The test results of IgY against bacteria *S. alpha* and *S. aureus* were negative or did not form inhibition zones (Figure 2).

The ability of antibacterial activity in some IgY serum concentration immunized with *S. mutans* was obtained against *S. mutans*. The test results of Minimal Inhibitory Concentration (MIC) and inhibition zones of several concentrations of IgY against *S. mutans* were shown in Figure 3.

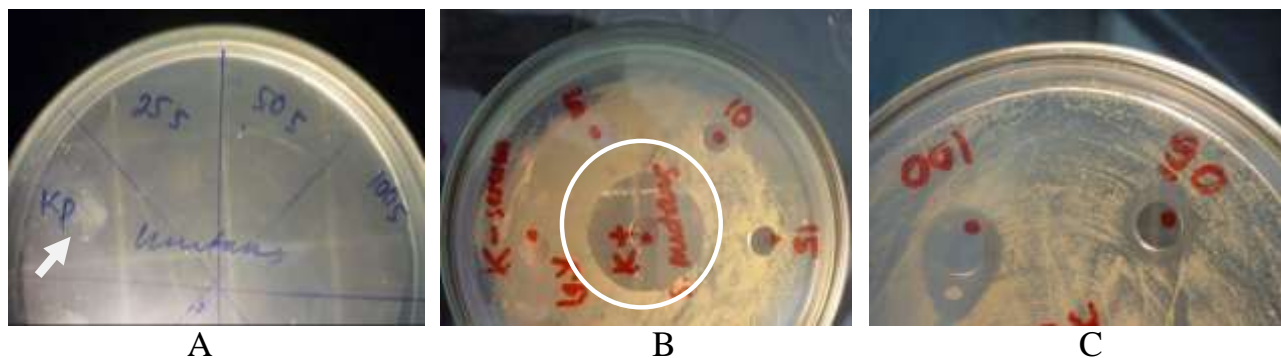
Measurement were done on the inhibition zone on 10 plate. In Figure 3 it can be seen that the minimum concentration of IgY *S. mutans* specific that has inhibition against *S. mutans* was a concentration of 25%. Highest inhibition zone were obtained on the positive control (chlorhexidine digluconate). On the solvent control or a negative control using distilled water did not form visible inhibition zone. Mean result ( $n = 10$ ) and standard deviation inhibition zone several concentrations of IgY *S. mutans* specific and control of *S. mutans* shown in Figure 4.

The Kolmogorov-Smirnov was used for normality calculation because the amount of data is 50. The result of the normality test showed  $p > 0.05$  or normal data distribution. Furthermore, homogeneity test showed  $p > 0.05$  or can be interpreted as homogeneous data. ANOVA was conducted to determine differences in inhibition zone of some concentrations of IgY serum *S. mutans* specific against *S. mutans* (Table 1).

Table 1 show that there was significant difference in



**Figure 2.** There was no inhibition zone the antibacterial activity of IgY serum against *S. alpha* (A) and *S. aureus* (B) by diffusion test. Inhibition zone formed on the positive control using chlorhexidine digluconate (circles) but not formed on the negative control using distilled water.



**Figure 3.** Antibacterial activity of IgY serum that has been immunized against *S. mutans*. The minimum concentration showed at 25% IgY against *S. mutans* (A). Inhibition zone was formed at a concentration of 50% and 100% (C) and a positive control (B) using chlorhexidine digluconate (circles) but not formed on the solvent control or negative control using distilled water (arrows).

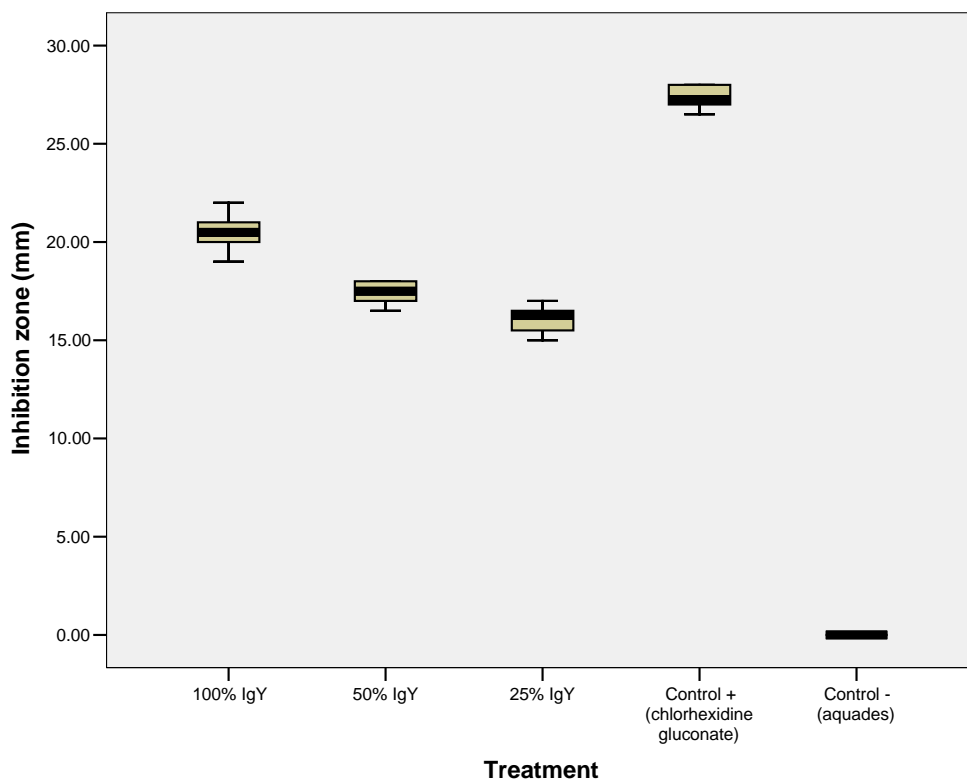
inhibition zones formed from several concentration of IgY *S. mutans* specific and control of *S. mutans*. Statistical testing was continued using LSD to compare each concentration as well as the control (Table 2).

In Table 2, it can be seen that all comparisons between treatment groups either concentrations of IgY serum *S. mutans* specific or IgY serum with control indicating  $p < 0.05$ . The results showed a significant difference between the ratio of concentrations of IgY serum *S. mutans* specific and control of the inhibition zone formed in *S. mutans*.

## DISCUSSION

The microorganisms found in the human oral cavity have been referred to as the oral microflora, oral microbiota, or more recently as the oral microbiome. Approximately,

280 bacterial species from the oral cavity have been isolated in culture and formally named. It has been estimated that less than half of the bacterial species present in the oral cavity can be cultivated using anaerobic microbiological methods and that there are likely 500 to 700 common oral species (Saini, 2015). The results showed immunization using antigen *S. mutans* for 4 weeks will result an antibody IgY serum specific *S. mutans* in the 5<sup>th</sup> week. It was aligned with previous study that IgY serum specific *S. mutans* in chicken egg yolk using Agar Gel Precipitation Test (AGPT) generated at week 5 (Azis et al., 2013). Bizanov and Jonauskiene (2003) also reported that after 2 weeks of initiation immunization, high immunospecific antibodies will be generated in the chicken egg yolk and that antibody titer will increase until the 7<sup>th</sup> week. Immunospecific antibodies will be actively transported from the serum to the chicken egg yolk during chicken pregnancy which immunized with



**Figure 4.** The mean inhibition zone of some concentrations IgY serum specific to *S. mutans* and control (n = 10). The highest results of inhibition zone (mm) were seen in the positive control. IgY serum inhibition zone increased along with increasing concentrations.

**Table 1.** ANOVA results showed inhibition zone some concentrations of IgY serum specific *S. mutans*.

Zone	Sum of squares	df	Mean square	F	Sig.
Between Groups	4065.850	4	1016.463	2647.804	0.000
Within Groups	17.275	45	0.384		
Total	4083.125	49			

**Table 2.** Summary of LSD test showed comparison inhibition zone (mm) between several concentrations of IgY serum specific *S. mutans* and control.

Concentration	25% IgY	50% IgY	100% IgY	Control +	Control -
25% IgY	-	0,000	0,000	0,000	0,000
50% IgY	0,000	-	0,000	0,000	0,000
100% IgY	0,000	0,000	-	0,000	0,000
control +	0,000	0,000	0,000	-	0,000
control -	0,000	0,000	0,000	0,000	-

specific antigens. Antibody specific *S. mutans* in chicken serum which was detected using ELISA appears first in 7

days after immunization and then detected in chicken egg yolk after 2 weeks. Specific antibodies titer will increase

in the chicken serum and reach the peak on day 45 and will remain stable until day 168 (Rajan et al., 2011). The results support previous study that the resulting IgY serum is specific to a particular immunized antigen (Rajan et al., 2011; Dinesh et al., 2013). It can be shown in Figure 2 that the IgY serum only has antibacterial activity against *S. mutans* and not against *S. aureus* and *S. alpha*. *S. mutans* is referred as the causative agent of caries through three stages, beginning with interactions with tooth surface mediated by adhesion, accumulation of bacteria in biofilm and the production of glucose and glucan by glucosyltransferase enzyme of bacteria, as well as the formation of lactic acid. Glucosyltransferase enzyme in bacteria is able to synthesize extracellular polysaccharides, especially hydrophobic glucan from sucrose and its ability to colonize with the tooth surfaces (Koo et al., 2010).

Figures 2 and 3 show the IgY serum *S. mutans* specific has antibacterial activity against *S. mutans*. Mechanism of antibacterial activity of the IgY *S. mutans* specific is suspected because of the specific IgY serum can weaken the pathogenicity of *S. mutans*. This is consistent with previous studies that IgY *S. mutans* specific could be expected to prevent dental caries by means of bacteria immobilization and demolish the bacteria's ability to convert sugar into acid. Prevention of adhesion of *S. mutans* to tooth surfaces can be done by using an antibody that has a target to block an antigenic adhesion of pathogens (Rajan et al., 2011). The ability of IgY specific to bind to the pathogenic bacteria will change the bacteria surface that can weaken the bacteria to attach (adhere) to the host cell. Hamajima et al. (2007) mentions that specific IgY can bind to the outer membrane proteins (outer membrane protein / OMP) of *P. gingivalis* bacteria that inhibit these bacteria to conduct co-aggregation. Other mechanism mentioned that a high IgY antibody titer will have great purity and effectively to neutralize various antigens *in vitro* and *in vivo*. As noted in the study of Meenatchisundaram et al. (2008) that IgY egg yolk has the ability to neutralize toxins from cobra. Other study mentions that IgY *S. aureus* specific given as an infusion into the mammary gland of cattle could inhibit the growth of *S. aureus* as a cause of mastitis.

Positive control using mouthwash ingredient chlorhexidine digluconate (CHX) showed antibacterial activity against *S. mutans*, *S. aureus* and *S. alpha* higher than IgY serum treatment. This is due to differences in the mechanism of antibacterial activity of both ingredients. IgY serum *S. mutans* specific has neutralization mechanism or weaken the ability of bacteria by binding to the bacteria and does not have the ability to kill bacteria while CHX has a toxic ability towards the cell despite of the low toxicity. In addition CHX containing bis-biguanide biocide cationic is known to have broad spectrum of antibacterial effect which causing disruption of bacterial cell membranes and death of cells. CHX cationic nature

allows it to bind to the tooth surface and oral mucosa that inhibits the formation of dental plaque and prevent gingivitis. Rinse using CHX for 5 days can reduce the number of *S. mutans* between 30-50% and the repeated use of mouthwash that can lead to changes in the oral flora ecosystem (McBain et al., 2003).

Distilled water is used for negative control or solvent in this study. The result shows the negative control has no antibacterial activity at all three bacteria tested. It can be concluded that the immunization of *S. mutans* produces IgY specific *S. mutans* serum and IgY specific *S. mutans* serum has antibacterial activity against *S. mutans* only.

### Conflict of interest

The authors declare that they have no conflict of interest

### ACKNOWLEDGEMENT

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